CHIMERIC PROTEIN FOR THE SCREENING OF AGONISTS AND ANTAGONISTS OF CELL SIGNALING PATHWAYS THAT ARE DEPENDENT ON G-PROTEIN-COUPLED RECEPTORS

The present invention relates to a recombinant chimeric protein derived from the α_1 and β subunits of high-threshold calcium channels, and also to its applications for studying G-protein-coupled receptor (GPCR)-dependent cell signaling pathways and identifying compounds that modulate G protein activity.

The GPCR class comprises more than a thousand identified members, encoded by genes representing 2 to 5% of the coding potential of the vertebrate genome (El Far and Betz, Biochem. J., 2002, 365, 329-336); there are 27 genes encoding Gα subunits, 5 encoding the Gβ subunits, and 14 encoding the Gγ subunits (Albert and Robillard, Cell, 2002, 14, 407-418).

Many biological processes, such as synaptic regulation, response to hormones and to pheromones, cell guiding (chemoattraction or chemorepulsion) or vision, involve G-protein-coupled receptors. In fact, GPCRs are capable of providing the recognition and the translation of messages as varied as those of amino acids (glutamic acid, etc.), peptides (angiotensin, neurotensin, somatostatin, etc.), proteins (thyrotropin (TSH), follicle-stimulating hormone (FSH), etc.), amines (acetylcholine, adrenaline, serotonin, etc.), lipids (prostaglandins, leukotrienes, etc.), nucleotides and nucleosides (adenosine or ATP). Ions (Ca^{++}), olfactory and taste molecules, photons and pheromones are also part of the extracellular signals recognized by GPCRs (for review, see Gether, Endocrine reviews, 2000, 21, 90-113 and Albert and Robillard, mentioned above).

The extracellular signal is transduced inside the cell by means of heterotrimeric G proteins that bind guanyl nucleotides (GDP and GTP), made up of subunits called

Gα, Gβ, Gγ; recognition of the extracellular signal by the GPCR leads to activation of the G proteins, which results in dissociation of the heterotrimer to Gα, and Gβγ, and binding of the Gα subunit to GTP.

Several intracellular effectors can be directly or indirectly modulated through activation of the various Gα and Gβγ subunits of G proteins. The effectors controlled by the Gα subunits may be enzymes (phospholipases A2 and C, adenylyl- and guanylylcyclases, c-jun kinase, tyrosine phosphatase (SH-PTP2), etc.), the activation of which will influence the amount of second messengers produced or released (phosphoinositides and diacyl glycerols, Ca^{++}, cAMP, cGMP, etc.), channels (potassium-, calcium-, sodium- or chlorine-conducting), ion exchangers (sodium/proton) or, more recently, kinases (Btk tyrosine kinases (Bruton's tyrosine kinase), MAP kinases (mitogen-activated protein kinase)) (Albert and Robillard, mentioned above).

Gβγ can also modify the activity of at least as many effectors as those controlled by Gα, namely: channels (voltage-dependent sodium-conducting or calcium-conducting channels (N and P/Q) or inward rectifier potassium channels (GIRK: G protein inward rectifier K+ channel), etc.), "conventional" enzymes (phospholipases A2 and C, adenylyl cyclase I, II and IV, tyrosine phosphatase (SH-PTP1), etc.), and also a considerable number of kinases (phosphoinositide 3-kinase, β-adrenergic receptor kinases, c-jun kinase, MAP kinases, Btk tyrosine kinases and T-cell-specific kinase (Tsk) (for review, see Albert and Robillard, mentioned above).

Thus, the study of these signaling pathways and the search for drugs that act on these signaling pathways is of considerable therapeutic interest in the search for novel medicinal products.

It emerges from the above that the activation of G proteins and the dissociation thereof into Gα and Gβγ subunits is the crossroads for a large number of cell signaling and regulatory pathways. Consequently, analysis of the activation of these G proteins makes it possible to study G-protein-coupled receptor-dependent cell signaling pathways and to screen agonists and antagonists of these signaling pathways.

To analyze the activation of G proteins, Janetopoulos et al. (Science, 2001, 291, 2408-2411) have described a technique for the real-time monitoring, *in vivo*, of the Gα-Gβ interaction. This technique, developed in the amoeba *Dictyostelium discoideum*, is based on the cotransfection of two constructs encoding two fluorescent chimeric proteins: Gβ-YFP and $G\alpha_2$-CFP. The interaction between the two chimeras induces a fluorescence transfer process (FRET), which makes it possible to follow, in real time, their interaction in the living cell. The two chimeras constructed by Janetopoulos et al. are capable of forming a complex that interacts functionally with the cAMP receptor and can be activated by GTPγS. This technique using fluorescence can be adapted to high throughput screening approaches. However, due to the competition with endogenous Gα or Gβγ subunits that suppresses the FRET process, this approach can only function in cells that have had their endogenous equivalent G protein genetically deleted. In addition, in vertebrates, the various isoforms of G proteins are involved in the response to activation of the various GPCR-type receptors. Consequently, the approach of Janetopoulos et al. presumes the construction of a chimera and of a cell line specifically deleted for each isotype of G protein studied.

It emerges from the above that no ubiquitous tool that is simple to use exists for evaluating G protein

activation in eukaryotic cells.

Calcium channels comprise low-threshold channels that are activated by weak depolarizations, and high-threshold channels that are activated by strong depolarizations. The high-threshold channels represent a heteromultimeric complex $\alpha_1\alpha_2\delta\beta$ and γ, in which the membrane-bound α_1 subunit, constituting the channel per se, is associated with an intracellular regulatory β subunit (or $Ca_v\beta$) via its interaction domain (AID domain for alpha interaction domain), having a conserved motif: **QQ-E--L-GY--WI---E** (one-letter code: - representing any amino acid; Pragnell et al., Nature, 1994, 368, 67-70; figure 1) in which residues Y392, W395 and I396 are essential for the biding of the β subunit (De Waard et al., FEBS, 1996, 380, 272-276). The regulatory β subunit binds to the AID domain by its BID domain (beta interaction domain; De Waard et al., J. Biol. Chem., 1995, 270, 12056-12064), which is included in a GK-like domain (Hanlon et al., FEBS, 1999, 445, 366-370).

Seven α_1 subunits have been identified: $\alpha_{1A}(Ca_v\alpha_{2.1})$, α_{1B} $(Ca_v\alpha_{2.2})$, α_{1E} $(Ca_v\alpha_{2.3})$, that form respectively the neuronal channels of P/Q and N type and the channels of R type, regulated by G proteins (G protein-sensitive channels), and $\alpha_{1S}(Ca_v\alpha_{1.1})$, $\alpha_{1c}(Ca_v\alpha_{1.2})$, $\alpha_{1d}(Ca_v\alpha_{1.3})$ and $\alpha_{1f}(Ca_v\alpha_{1.4})$, that form the G protein-insensitive L-type channels, including the cardiovascular channels (α_{1c}) and skeletal channels (α_{1S}) (Lory et al., m/s, 2001, 10, 979-988).

In the central nervous system, the N- and P/Q-type high-threshold calcium channels are directly involved in the triggering of synaptic function; the opening thereof under the effect of an action potential induces calcium entry into the presynaptic terminal. This signal triggers the secretion of neuromediators such as glutamate into the synaptic cleft, and thus the

propagation of the nervous influx in the postsynaptic dendrite. N and P/Q channels are regulated by trimeric G-protein-coupled receptors (GPCRs) such as class III metabotropic glutamate receptors (for review: El Far and Betz, mentioned above) or noradrenergic, muscarinic, GABAergic (GABA: 5-γ-aminobutyric acid), serotoninergic or dopaminergic receptors, and opiate receptors (for review: Hille, Trends NeuroSci., 1994, 17, 531-536). It has been shown that the Gβγ subcomplex is directly responsible for inhibition of the activity of P/Q channels that results from direct binding of Gβγ to the intracytoplasmic loop connecting membrane domains I and II (loop I-II) of the α_1 subunit (De Waard et al., Nature, 1997, 385, 446-450). As a result, this loop has several sites of interaction with Gβγ, that overlap the $Ca_v\beta$ regulatory subunit-binding domain (AID domain; figure 1) a consensus motif **QQ--R-L-GY** of which, included in the AID domain, is essential for the binding of Gβγ (figure 1; De Waard et al., Nature 1997, 385, 446-450; Zamponi et al., Nature, 1997, 385, 442-446). Furthermore, the $Ca_v\beta$ regulatory subunit appears to oppose the functional effect of G proteins (Bourinet et al., P.N.A.S., 1996, 93, 1486-1491). Thus, it would appear that this antagonism involves physical competition between the $Ca_v\beta$ subunit and the Gβγ protein at the AID region of the I-II loop (Dolphin et al., J. Physiol., 1998, 506, 3-11).

The inventors have constructed chimeric proteins by NH_2- and/or COOH-terminal fusion:

(i) of the I-II loop of the α_1 subunit of G-protein-sensitive or -insensitive high-threshold calcium channels (respectively, α_{1A} or $Ca_v\alpha_{2.1}$ constituting a P/Q-type neuronal channel, and α_{1c} or $Ca_v\alpha_{1.2}$ constituting an L-type cardiovascular channel) or a fragment thereof; said loop corresponds to positions 367 to 487 with reference to the sequence of the $Ca_v\alpha_{2.1}$ subunit, and comprises the

domain for binding to a β subunit of a calcium channel (or AID domain) and the sites for binding to a Gβ subunit of a G protein (figure 1), and

(ii) of a β subunit of a high-threshold calcium channel, capable of binding to said fragment of the α_1 subunit.

They have shown that all the chimeric proteins obtained, comprising the I-II loop of an α_1 subunit derived from a G-protein-sensitive or -insensitive calcium channel or a fragment thereof including at least the AID domain, have surprising properties of intramolecular interaction between the binding domains of the α_1 and β subunits of the calcium channel, that prevent the binding of the chimera to the AID domain of an α_1 subunit. They have confirmed that this masking of the binding domain of the β subunit was indeed due to its intramolecular interaction with the AID domain, since deletion of the AID domain from the chimera re-establishes this binding. They have also shown that the binding of the chimera comprising the I-II loop of a "G-protein-sensitive" α_{1A} subunit, to the α_1 subunit interaction domain is re-established by the addition of Gβγ. This result was confirmed by the demonstration, *ex vivo*, of the interaction of a recombinant β subunit labeled with a Cy3-type fluorophore, with a fluorescent chimera of the α_{1A} subunit (GFP-α_{1A}), by means of fluorescence transfer (FRET) measurement using confocal microscopy. These properties have allowed them to demonstrate that, unexpectedly, the regulation of P/Q channels involves a displacement, by the Gβγ complex, of the interaction between the regulatory β subunit and the α subunit of the calcium channel, and not its inhibition, as had been previously suggested.

More precisely, the inventors have shown that the chimeric protein derived from a G-protein-sensitive α_1 subunit exists in two forms, that are "closed" or

"open", respectively in the absence or in the presence of a Gβ subunit capable of binding to said fragment of the α_1 subunit, either in the form of a Gβ monomer or in the form of a Gβγ heterodimer. In the absence of Gβ (or Gβγ), the chimeric protein is capable of folding on itself, thus allowing the interaction domains of the α_1 and β subunits of the calcium channel to associate by means of a stable intramolecular binding (closed form). In the presence of Gβ (or Gβγ), the intramolecular binding is destroyed and the interaction domains of the α_1 and β subunits of the calcium channel dissociate (open form), thus allowing each of the domains to interact, respectively, with Gβ (α_1 subunit interaction domain: AID domain) and/or an α_1 subunit of a calcium channel (β subunit interaction domain: BID domain).

Similarly, the chimeric protein derived from a G-protein-insensitive α_1 subunit is capable of folding on itself, thus allowing the interaction domains of the α_1 and β subunits of the calcium channel to associate by means of a stable intramolecular binding (closed form). Consequently, in the presence of antagonists for this binding, other than Gβ or Gβγ, the intramolecular binding can also be destroyed and the interaction domains of the α_1 and β subunits of the calcium channel dissociate (open form), thus allowing each of the domains to interact, respectively, with said antagonist other than Gβ or Gβγ (α_1 subunit interaction domain: AID domain) and/or an α_1 subunit of a calcium channel (β subunit interaction domain: BID domain).

Consequently, due to the reorganization of their structure in the presence of free Gβ or Gβγ subunits or else other antagonists of the interaction between the α_1 and β subunits (change from the closed form to the open form), the chimeric proteins derived from the α_1 and β subunits of high-threshold calcium channels represent sensitive, specific tools that are simple to use, and are useful for the following applications:

- the chimeric proteins derived from a G-protein-sensitive α_1 subunit (for example: α_{1A}, α_{1B} and α_{1E}) make it possible to determine the variations in cellular concentration of free $G\beta\gamma$ subunits, *ex vivo*, in real time and therefore to measure the activation of G proteins in cells: such chimeric proteins represent ubiquitous biosensors for G protein activation that are entirely suitable for studying G-protein-coupled receptor-dependent cell signaling and regulatory pathways and for screening agonists/antagonists of these signaling pathways that are capable of increasing or of decreasing the concentration of free $G\beta\gamma$ subunits in cells and therefore of modulating the activity of these G-protein-coupled receptor-dependent cell signaling and regulatory pathways;

- the chimeric proteins derived from a G-protein-sensitive or -resistant α_1 subunit (for example: α_{1A}, α_{1B}, α_{1E}, α_{1c}, α_{1d}, α_{1S} and α_{1f}) represent simple, sensitive and specific tools that are entirely suitable for screening antagonists of the interaction between the α_1 and β subunits, that are capable of modulating the activity of all high-threshold calcium channels;

- the chimeric proteins derived from an α_1 subunit and from a β subunit of a high-threshold calcium channel, as defined above, are also useful for the systematic pharmacotoxicological control of new medicinal products in phase I and the search for natural agonists of orphan receptors. In fact, the cloning of the human genome has made it possible to identify approximately 350 GPCR receptors, Among these, only 200 have an identified ligand. The others, called orphan receptors, potentially constitute key targets for the identification of novel cell signaling and regulatory pathways. The

search for agonists and for antagonists of these receptors is therefore of major interest both from the point of view of fundamental research and from a therapeutic point of view.

Consequently, a subject of the present invention is a chimeric protein derived from a high-threshold calcium channel, characterized in that it comprises at least one β subunit or a fragment thereof including at least the BID domain, fused, at its NH_2 or COOH end, with the I-II loop of an α_1 subunit or a fragment thereof including at least the AID domain.

In accordance with the invention, the AID and BID domains are as defined above; the I-II loop of the α_1 subunit comprises the AID domain for binding to the β subunit and the sites for binding to the Gβ subunit of a G protein, including a consensus binding site that is included in this AID domain. These various domains are illustrated in figure 1.

The invention encompasses the chimeric proteins derived from the α_1 and β subunits of vertebrates, in particular of human or nonhuman mammals and of their orthologs in invertebrates.

Chimeric proteins in accordance with the invention are represented in particular by:

- a β subunit fused, at its NH_2 or COOH end, with the I-II loop of an α_1 subunit, and

- the GK-like domain of a β subunit including the BID domain (Hanlon et al., mentioned above), fused, at its NH_2 or COOH end, with the I-II loop of an α_1 subunit.

In accordance with the invention, the I-II loop, or a fragment thereof, is either fused directly to the NH_2

or COOH end of the β subunit or of a fragment thereof, or the two sequences are separated by means of a spacer peptide whose size and amino acid sequence are such that the AID and BID domains of the chimeric protein containing said spacer are capable of interacting so as to form intramolecular binding that is displaced in the presence of an antagonist (change from the closed form to the open form); such a spacer peptide is in particular represented by a polyglycine sequence.

According to an advantageous embodiment of said chimeric protein, it is derived from a G-protein-sensitive high-threshold calcium channel.

According to an advantageous provision of this embodiment, said chimeric protein comprises a fragment of an α_1 subunit selected from α_{1A}, α_{1B} and α_{1E}.

According to another advantageous embodiment of said chimeric protein, said β subunit is selected from the group consisting of β_1, β_2, β_3 and β_4.

The invention also encompasses the chimeric proteins consisting of sequences that are functionally equivalent to the sequences as defined above, i.e. in which the β subunit and the I-II loop of the α_1 subunit or fragments thereof as defined above are capable of forming intramolecular binding by means of their interaction domains; said binding being optionally destroyed in the presence of free Gβ or Gβγ subunits or else other antagonists of the interaction between the α_1 and β subunits ("open form").

Among these sequences, mention may, for example, be made of the sequences derived from the preceding sequences by:

- mutation (substitution and/or deletion and/or addition) of one or more amino acids of the sequences as defined above,

- modification of at least one -CO-NH- peptide bond of the peptide chain of the chimeric protein as defined above, in particular by replacement with a bond different from the -CO-NH- bond (methyleneamino, carba, ketomethylene, thioamide, etc.) or by introduction of a retro-type or retro-inverso-type bond, and/or

- substitution of at least one amino acid of the peptide chain of the chimeric protein as defined above, with a nonproteinogenic amino acid residue.

The term "nonproteinogenic amino acid residue" is intended to mean any amino acid that is not part of the constitution of a natural protein or peptide, in particular any amino acid in which the carbon bearing the side chain R, namely the -CHR- group, located between -CO- and -NH- in the natural peptide chain, is replaced with a motif that is not part of the constitution of a natural protein or peptide.

A subject of the present invention is in particular a variant chimeric protein derived from a chimeric protein as defined above, characterized in that it has a mutation of at least one amino acid in the sequences of said β subunit and/or of the I-II loop of an α_1 subunit.

According to another advantageous embodiment of said chimeric protein, said variant has a mutation that modifies the affinity of the β subunit for the I-II loop of the α_1 subunit and/or vice versa; such mutations make it possible to obtain a chimeric protein that is more or less sensitive to the concentration of free $G\beta$ or $G\beta\gamma$ subunits.

Among these mutations, mention may be made of mutations of the AID domain of the I-II loop of the α_1 subunit, as described in Pragnell et al., mentioned above, and De Waard et al., FEBS, 1996, 380, 272-276, i.e.: Q383A, Q384A, E386D, E386S, L389H, G391R, Y392S, Y392F, W395A, I396A and E400A.

According to another advantageous embodiment of said chimeric protein or of its variant, it is coupled, preferably covalently, to at least one suitable label allowing the detection and/or the purification and/or the immobilization of said protein, for example: an antigenic epitope, a polyhistidine-type tag, or a luminescent compound (fluorophore such as GFP or one of its variants: CFP, YFP and BFP), a radioactive compound or an enzymatic compound.

In accordance with the invention, said coupling is carried out by any appropriate means, in particular via a peptide bond by means of the COOH- and/or NH_2-terminal functions of the peptide chain, or else via another covalent bond, for instance: an ester, ether, thioether or thioester bond, by means of reactive functions of the side chain of an amino acid of the peptide chain.

According to an advantageous provision of this embodiment, said chimeric protein comprises an acceptor or donor fluorophore respectively at its NH_2 and/or COOH end.

The acceptor fluorophores, for example CFP or BFP, can be coupled without distinction at the NH_2 or COOH end of the chimeric protein, the donor fluorophores, for example CFP or YFP, are fused to the opposite end of said chimeric protein. Such chimeric proteins are useful for the real-time *ex vivo* study of G protein activation and the screening of molecules capable of

modulating this activation, by measurement of fluorescence transfer (FRET).

In fact, the labeling with a luminescent compound has the advantage of obtaining a localized signal that does not require the presence of other reagents, as is the case for enzymatic labelings. This type of labeling also makes it possible to use a phenomenon such as energy transfer that can take place according to various mechanisms: resonance energy transfer, radiative energy transfer (the acceptor absorbs the light emitted by the donor) and electron transfer.

This energy transfer, between a luminescent "donor" compound (D) and a luminescent or nonluminescent "acceptor" compound (A), and which depends on the distance between A and D, has been used for carrying out many assays. D and A, which are coupled to each end of the chimeric protein so that the energy transfer takes place only when the intramolecular interaction between the BID and AID domains takes place (closed form), are chosen. This phenomenon results in a decrease or quenching of the luminescence of D and an emission of luminescence from A if the latter is luminescent, when D is excited. During these assays, either the variation in luminescence of A or the variation in luminescence of D is measured, the nature of A and of D being variable. For example, to measure the variation in luminescence of A, two fluorescent proteins can be used as donor and acceptor, or else a complex of rare earth metals (europium, terbium) with a chelate, a cryptate or a macrocycle can be used as donor and a fluorescent protein can be used as acceptor. The measurement of the variation in luminescence of D is based on the ability of a compound (A) to decrease or eliminate the luminescence of another compound (D) when they are sufficiently close ("quench"). The range of molecules A that can be used is therefore broader and thus includes nonluminescent

compounds such as heavy metal, heavy atoms, chemical molecules, for instance methyl red, nanoparticles such as those sold under the name Nanogold® by the company Nanoprobes (USA), or else the molecules sold under the names DABCYL® (Eurogentec, Belgium), QSY Dyes (Molecular Probes Inc., USA), ElleQuencher® (Oswell/Eurogentec) or Black Hole Quenchers® (Biosearch Technologies Inc., USA).

A subject of the present invention is also a peptide, characterized in that it comprises a fragment of at least 7 amino acids of the sequence of the chimeric protein as defined above, located at the junction of the β subunit and of the I-II loop of the α_1 subunit or of their fragments as defined above; such peptides make it possible in particular to produce antibodies specific for the chimeric protein according to the invention.

A subject of the present invention is also antibodies, characterized in that they are directed against a chimeric protein or a peptide as defined above.

In accordance with the invention, said antibodies are either monoclonal antibodies or polyclonal antibodies.

These antibodies can be obtained by conventional methods, that are known in themselves, comprising in particular the immunization of an animal with a protein or a peptide in accordance with the invention, in order to make it produce antibodies directed against said protein or said peptide.

Such antibodies are useful in particular for immobilizing the chimeric protein on a solid support, purifying it, or else detecting it.

A subject of the present invention is also a nucleic acid molecule, characterized in that it is selected

from the group consisting of the sequences encoding a chimeric protein or a peptide as defined above and the sequences complementary to the above sequences, that may be sense or antisense.

A subject of the invention is also probes and primers, characterized in that they comprise a sequence of approximately 10 to 30 nucleotides corresponding to that located at the junction of the β subunit and of the I-II loop of the α_1 subunit or of their fragments as defined above; these probes and these primers make it possible to specifically detect/amplify said nucleic acid molecules encoding the chimeric protein according to the invention.

A subject of the invention is also other primers for specifically amplifying the β subunit and/or the I-II loop of the α_1 subunit or their fragments as defined above, characterized in that they are selected from the group consisting of the sequences SEQ ID NO: 1, 2, 4, 6, 7, 8 and 9.

The nucleic acid molecules according to the invention are obtained by conventional methods, that are known in themselves, according to standard protocols such as those described in Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA).

The sequences encoding a chimeric protein according to the invention can be obtained by amplification of a nucleic acid sequence by PCR or RT-PCR using a suitable pair of primers, or else by screening genomic DNA libraries by hybridization with a homologous probe.

The derived nucleic acid molecules, encoding a variant of the chimeric protein according to the invention, are obtained by conventional methods for introducing mutations into a nucleic acid sequence, that are known

in themselves, according to the abovementioned standard protocols. For example, the sequence encoding a variant of the chimeric protein according to the invention can be obtained by site-directed mutagenesis according to the method of Kunkel et al. (P.N.A.S., 1985, 82, 488-492).

A subject of the present invention is also a recombinant eukaryotic or prokaryotic vector, characterized in that it comprises an insert consisting of the nucleic acid molecules encoding a chimeric protein as defined above.

Preferably said recombinant vector is an expression vector in which said nucleic acid molecule or one of its fragments is placed under the control of suitable regulatory elements for transcription and translation. In addition, said vector can comprise sequences fused in frame with the 5' and/or 3' end of said insert, that are useful for immobilizing and/or detecting and/or purifying the protein expressed from said vector. Many vectors into which it is possible to insert a nucleic acid molecule of interest in order to introduce it into and to maintain it in a eukaryotic or prokaryotic host cell are known in themselves; the choice of a suitable vector depends on the use envisioned for this vector (for example, replication of the sequence of interest, expression of this sequence, maintenance of the sequence in extrachromosomal form or else integration into the host's chromosomal material), and also on the nature of the host cell. For example, viral or nonviral vectors such as plasmids can be used.

These vectors are constructed and introduced into host cells by conventional recombinant DNA and genetic engineering methods that are known in themselves.

According to one embodiment of said recombinant vector, it is a eukaryotic expression vector having a sequence

selected from the group consisting of the sequences SEQ ID NO: 5 and SEQ ID NO: 10; the plasmid SEQ ID NO: 5 contains the I-II loop of the rabbit $Ca_v\alpha_{2.1}$ subunit, fused to the C-terminal end of a rat $Ca_v\beta_3$ subunit, under the control of the CMV promoter, and the plasmid SEQ ID NO: 10 contains an insert consisting, from 5' to 3', of the in-frame fusion of the following fragments: the sequence GAP-43, the cDNA encoding EGFP (fluorescence donor), the GK-like domain of the rat $Ca_v\beta_3$ subunit, the I-II loop of the rabbit $Ca_v\alpha_{2.1}$ subunit and the cDNA encoding CFP (fluorescence acceptor).

A subject of the present invention is also cells modified with a chimeric protein, a nucleic acid molecule or else a recombinant vector as defined above.

According to an advantageous embodiment of the invention, said cells are eukaryotic cells.

According to an advantageous provision of this embodiment, said cells express at least one G-protein-coupling receptor (GPCR); said cells are either cells constitutively expressing at least one GPCR, or modified cells that express a recombinant GPCR.

Modified cells in accordance with the invention can be obtained by any means, that are known in themselves, for introducing a nucleic acid molecule or a protein into a host cell. For example, in the case of animal cells, use may be made, inter alia, of viral vectors such as adenoviruses, retroviruses, lentiviruses and AAVs, into which the sequence of interest has been inserted beforehand; said nucleotide sequence (isolated or inserted into a plasmid vector) or peptide sequence can also be combined with a substance that allows it to cross the host-cell membrane, for example a preparation of liposomes, of lipids or of cationic polymers, or else it can be injected directly into the host cell.

A subject of the present invention is nonhuman transgenic animals and in particular mammals, characterized in that all or some of their cells are transformed with a nucleic acid molecule according to the invention. They are, for example, animals into which a sequence encoding the chimeric protein according to the invention, under the control of suitable regulatory elements for transcription and translation, has been introduced. Such transgenic animals are useful in particular for the secondary screening steps: i) for evaluating the cell, or even tissue, targeting of a molecule active on GPCRs or calcium channels, that was identified in a primary screen, ii) for studying the bioavailability of such a molecule, and iii) for investigating, in a first approach, possible side effects of such a molecule.

The subject of the present invention is also the use of a product selected from the group consisting of the chimeric proteins, the nucleic acid molecules, the recombinant vectors, the modified cells and the nonhuman transgenic mammals as defined above, for studying G-protein-coupled receptor-dependent cell signaling and regulatory pathways.

A subject of the present invention is also the use of a product selected from the group consisting of the chimeric proteins, the nucleic acid molecules, the recombinant vectors, the modified cells and the nonhuman transgenic mammals as defined above, for screening agonists and/or antagonists of G-protein-coupled receptor-dependent cell signaling and regulatory pathways.

A subject of the present invention is also the use of a product selected from the group consisting of the chimeric proteins, the nucleic acid molecules, the recombinant vectors, the modified cells and the

nonhuman transgenic mammals as defined above, for screening antagonists of the interaction between the α_1 and β subunits of high-threshold calcium channels; such antagonists are useful for modulating the activity of all high-threshold calcium channels and therefore represent medicinal products that can be used in the treatment of diseases associated with a dysfunction of calcium homeostasis and of pathologies where modulation of calcium entry can compensate for a cellular deficiency, in particular epilepsy, ataxia, migraines, hypo- and hypercalcemia in the muscles, diabetes, and cardiovascular diseases.

According to an advantageous embodiment of the invention, the study of the G-protein-coupled receptor-dependent cell signaling and regulatory pathways is carried out by means of a method comprising at least the following steps:

a_1) culturing of modified cells expressing a chimeric protein derived from a G-protein-sensitive calcium channel and a G-protein-coupled receptor, as defined above,

b_1) transduction of a signal via said G-protein-coupled receptor, by any appropriate means, and

c_1) determination, by any appropriate means, of the proportion of said chimeric protein expressed in said cells that is bound to a $G\beta\gamma$ subunit.

Such a determination makes it possible to evaluate the variations in cellular concentration of free $G\beta\gamma$ subunits and therefore to measure the G protein activation in the cells.

According to an advantageous embodiment of the invention, the screening of agonists/of antagonists of the G-protein-coupled receptor-dependent cell signaling

and regulatory pathways is carried out by means of a method comprising at least the following steps:

a_2) culturing of modified cells expressing a chimeric protein derived from a G-protein-sensitive calcium channel and a G-protein-coupled receptor, as defined above,

b_2) transduction of a signal via said G-protein-coupled receptor, by any appropriate means,

c_2) comparative determination, by any appropriate means, of the proportion of said chimeric protein expressed in the cells that is bound to a $G\beta\gamma$ subunit, before and after the bringing into contact of said cells in b_2) with a molecule to be tested, and

d_2) identification of the molecules that are agonists/antagonists of the G-protein-coupled receptor-dependent cell signaling and regulatory pathways, corresponding to those capable respectively of increasing and of decreasing the cellular concentration of free $G\beta\gamma$ subunits.

Advantageously, said modified cells in a_1) or in a_2) express a chimeric protein as defined above coupled, at its NH_2 and COOH ends, respectively to a fluorescence donor fluorophore and a fluorescence acceptor fluorophore, and said determination in c_1) or in c_2) is carried out by means of the fluorescence transfer (FRET) technique.

According to an advantageous embodiment of the invention, the screening of antagonists of the interaction between the α_1 and β subunits of high-threshold calcium channels is carried out by means of a method comprising at least the following steps:

a_3) bringing a molecule to be tested into contact with a chimeric protein derived from a G-protein-sensitive or -insensitive calcium channel as defined above and with a peptide comprising the AID domain of a G-protein-insensitive α_1 subunit,

b_3) measuring, by any appropriate means, the binding of said chimeric protein to said peptide, and

c_3) identifying the antagonists of the interaction between the α_1 and β subunits corresponding to those with which binding of said chimeric protein to said peptide is observed.

According to an advantageous embodiment of said method, said peptide comprising the AID domain is immobilized on a solid support, and said chimeric protein is coupled to a label for measuring said binding in b_3), as defined above, in particular a fluorophore.

A subject of the invention is also a kit for implementing the methods as defined above, characterized in that it includes at least one of the following products: a chimeric protein, an antibody, a recombinant vector, a modified cell or a nonhuman transgenic mammal, as defined above.

The chimeric protein of the invention has the following advantages:

- it constitutes a ubiquitous biosensor for endogenous free $G\beta\gamma$ subunits that is suitable for the real-time study of G-protein-coupled receptor-dependent cell signaling and regulatory pathways, and for the systematic screening (high-throughput screening) of molecules capable of modulating them, that can potentially be used as a medicinal product for the treatment of diseases in which a dysfunction of these pathways is observed, in

particular immune system pathologies (for review, see Lombardi et al., Crit. Rev. Immunology, 2002, 22, 141-163; Onuffer and Horuk, Trends in Pharmacol, 2002, 23, 459-467) and neuropsychiatric and cardiovascular pathologies (Seifert and Wenzel-Seifert, Naumyn-Schmeideberg's Arch. Pharmacol., 2002, 366, 381-416). In addition, its use is simple insofar as it makes it possible to partially do away with problems of stoichiometry since its use involves only two molecules ($Ca_v\beta/Ca_v\alpha$-$G\beta\gamma$) instead of three partners ($Ca_v\alpha/Ca_v\beta/G\beta\gamma$) for the methods of the prior art;

- it is suitable for systematic screening (high-throughput screening) of molecules capable of modulating the activity of high-threshold calcium channels, that can potentially be used as a medicinal product for the treatment of diseases in which a dysfunction of calcium homeostasis is observed and of pathologies where the modulation of calcium entry can compensate for a cellular deficiency, as defined above.

Besides the above provisions, the invention also comprises other provisions which will emerge from the following description, which refers to examples of use of the chimeric protein that is the subject of the present invention, and also to the attached drawings, in which:

- figure 1 illustrates the overlap, in the I-II loop of the $Ca_v\alpha_{2.1}$ subunit, of the domains for binding to the β subunit (AID domain) and to the $G\beta\gamma$ complex. The AID domain is represented by a black box (positions 383 to 400). The binding sites for the $G\beta$ ($G\beta\gamma$) subunit are represented by hatched boxes; the site in the central position (**QQ--R-L-GY**) that is essential for the binding of the $G\beta$ ($G\beta\gamma$) subunit is included in the AID domain,

- figures 2 and 3 illustrate the displacement of the $Ca_v\alpha_{2.1}$-$Ca_v\beta$ interaction by the G-protein $G\beta\gamma$ complex:

- figure 2a illustrates the binding of the β_3 subunit (1 to 3 pM) with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein (1 µM),

- figure 2b shows that the fusion of the β_3 subunit with the I-II loop of the $\alpha_{2.1}$ subunit ($Ca_v\beta_3$-I-$II_{2.1}$ chimera) prevents its binding with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein,

- figure 2c shows that the deletion of the 18 amino acids of the $AID_{2.1}$ domain ($Ca_v\beta_3$-I-$II_{2.1}\Delta AID$ chimera) restores the binding of the β_3 subunit with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein,

- figure 3 shows that the addition of $G\beta\gamma$ complex displaces the intramolecular interaction between the $Ca_v\beta$ subunit and the I-II loop of the $\alpha_{2.1}$ subunit of the $Ca_v\beta_3$-I-$II_{2.1}$ chimera, thus allowing the β_3 subunit to bind with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein; the concentration of $G\beta\gamma$ capable of displacing 50% of the binding between the $Ca_v\beta$ subunit and the $AID_{2.1}$ domain (IC_{50}) is 160 nM,

- figures 4 to 7 illustrate the FRET analysis of the disassembly of the P/Q calcium channel, induced by the $G\beta\gamma$ complex:

- figure 4a illustrates the Cy3-labeling of the purified His-$Ca_v\beta_3$ subunit. CB: Coomassie blue staining of an SDS-PAGE gel illustrating the purity of the protein. FS = recording of the fluorescence of an unstained gel showing the

covalent labeling of the protein,

- figure 4b illustrates the effect of the $Ca_v\beta_3$ subunit coupled to a fluorochrome (Cy3-$Ca_v\beta_3$) on the current-voltage relationship of $Ca_v\alpha_{2.1}$ channels expressed in xenopus oocytes, by comparison with the unlabeled $Ca_v\beta_3$ subunit (injection of cRNA),

- figure 5a illustrates the observation by confocal microscopy of two distinct regions of xenopus oocytes containing $Ca_v\alpha_{2.1}$ and Cy3-$Ca_v\beta_3$. T = transmission, F = fluorescence,

- figure 5b illustrates the fluorescence emission spectrum for GFP-$Ca_v\alpha_{2.1}$, Cy3-$Ca_v\beta_3$ and (GFP-$Ca_v\alpha_{2.1}$ + Cy3-$Ca_v\beta_3$),

- figure 6 illustrates the kinetics of decrease in fluorescence transfer induced by the injection of 100 nM of $G\beta\gamma$. Upper panel: variations in the fluorescence emission spectrum, and lower panel: variations in the ratio of fluorescence intensities (R_f) at 585 nm and 525 nm,

- figure 7 illustrates the R_f values of the noninjected oocytes (-), oocytes injected with $G\beta\gamma$ (100 nM) or oocytes injected with heat-inactivated $G\beta\gamma$ (HI-$G\beta\gamma$),

- figure 8 (a to c) illustrates the sequence of the plasmid pcDNA3Cavβ3-I-$II_{2.1}$ (SEQ ID NO: 5) containing the I-II loop of the rabbit $C\alpha_v\alpha_{2.1}$ subunit fused to the C-terminal end of the rat $Ca_v\beta_3$ subunit, under the control of the CMV promoter,

- figure 9 (a to c) illustrates the sequence of the plasmid pCHIC (SEQ ID NO: 10) derived from the

vector pEYFPmemb. (CLONTECH), containing an insert consisting, from 5′ to 3′, of the in-frame fusion of the following fragments: the GAP-43 sequence, the cDNA encoding EGFP (fluorescence donor), the GK-like domain of the rat $Ca_v\beta_3$ subunit, the I-II loop of the rabbit $Ca_v\alpha_{2.1}$ subunit and the cDNA encoding CFP (fluorescence acceptor).

It should be clearly understood, however, that these examples are given only by way of illustration of the subject of the invention, of which they in no way constitute a limitation.

EXAMPLE 1: CONSTRUCTION OF A RECOMBINANT CHIMERIC PROTEIN Cavβ3-I-II$_{2.1}$

1) Materials and methods

The PCR amplification and the cloning of the recombinant DNA are carried out by conventional techniques known to those skilled in the art, according to standard protocols such as those described, for example, in Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA).

An expression plasmid containing a cDNA encoding a chimeric protein according to the invention, consisting of the C-terminal fusion of the rat β3 subunit with the I-II intracellular loop of the rabbit α_1 subunit, was constructed in the following way:

The cDNA of the rat $Ca_v\beta_3$ subunit (corresponding to positions 98 to 1545 of the Genbank sequence M88755) is amplified by PCR using the following sense and antisense primers:

- 5′-

TTTGGTACCATGGATGACGACTCCTACGTGCCCGGGTTTGAGGACTCGGAGGCGGGTT-3′ (SEQ ID NO: 1), and

- 5'-GCGGAATTCGTAGCTGTCCTTAGGCCAAGGCCGGTTACGCTGCCAGTT-3', (SEQ ID NO: 2).

The fragment thus obtained was cloned between the *Kpn I* and *EcoR I* sites of the expression plasmid (pcDNA3, Invitrogen), to give the recombinant plasmid pcDNA3-$Ca_v\beta 3$.

The cDNA fragment corresponding to the I-II loop of the rabbit $Ca_v\alpha_{2.1}$ subunit (positions 1383 to 1754 of the Genbank sequence X57477), the sequence of which is illustrated in figure 1, was amplified by PCR using the following sense and antisense primers:
- 5'-GGGGAATTCGCCAAAGAAAGGGAGCGGGTGGAGAAC-3'
(SEQ ID NO: 3; De Waard et al., mentioned above and Bichet et al., Neuron, 2001, 25, 177-190), and
- 5'-
TTTGAATTCTTACTGAGTTTTGACCATGCGACGGATGTAGAAACGCATTCT-3'
(SEQ ID NO: 4).

The fragment obtained was cloned into the *EcoR I* site of the plasmid pcDNA3-Cavβ3, to give the recombinant plasmid pcDNA3-$Ca_v\beta 3$-I-$II_{2.1}$.

A control plasmid containing a cDNA encoding a chimeric protein consisting of the C-terminal fusion of the rat β_3 subunit with the I-II intracellular loop of the rabbit $Ca_v\alpha_{2.1}$ subunit from which the AID domain had been deleted was constructed in a similar manner; the recombinant plasmid thus obtained was called pcDNA3-Cavβ_3-I-$II_{2.1}\Delta$AID.

2) Results

The recombinant plasmid pcDNA3-$Ca_v\beta 3$-I-$II_{2.1}$ has the sequence SEQ ID NO: 5. The peptide sequence deduced from the nucleotide sequence obtained by automatic sequencing of the insert cloned into the plasmid pcDNA3-$Ca_v\beta 3$-I-$II_{2.1}$ has the sequence expected for a

chimeric protein according to the invention. Similarly, the peptide sequence deduced from the nucleotide sequence obtained by automatic sequencing of the insert cloned into the plasmid pcDNA3-$Ca_v\beta 3$-I-$II_{2.1}\Delta$AID corresponds to that expected for a chimeric protein from which the AID domain has been deleted.

EXAMPLE 2: IN VITRO DEMONSTRATION OF THE DISPLACEMENT OF THE $Ca_v\alpha_{2.1}$-$Ca_v\beta$ INTERACTION BY THE G-PROTEIN G$\beta\gamma$ COMPLEX

1) Materials and methods

The expression of the recombinant DNA and the analysis of the recombinant proteins are carried out by conventional techniques known to those skilled in the art, according to standard protocols such as those described, for example, in Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA) and in Current protocols in Immunology (John E. Coligan, 2000, Wiley and son Inc, Library of Congress, USA).

a) Expression of the recombinant chimeric proteins and of the GST-$AID_{1.2}$ fusion

The chimeric proteins $Ca_v\beta 3$-I-$II_{2.1}$ and $Ca_v\beta 3$-I-$II_{2.1}\Delta$AID, and $Ca_v\beta_3$ subunit, are transcribed and translated *in vitro*, in the presence of [^{35}S]-methionine, from the plasmids as described in Example 1, using the Promega TNT system kit according to the supplier's instructions.

The GST-$AID_{1.2}$ fusion protein described in Pragnell et al., mentioned above, is produced and purified as described by the above authors. The GST protein produced and purified under the same conditions is used as a control.

b) *In vitro* analysis of the regulation of the $Ca_v\alpha_{2.1}$-$Ca_v\beta$ interaction by the G-protein $G\beta\gamma$ complex

The *in vitro* analysis of the regulation of the $Ca_v\alpha_{2.1}$-$Ca_v\beta$ interaction by the G-protein $G\beta\gamma$ complex is carried out according to the protocols as described in De Waard et al., Nature, 1997, 385, 446-450. More precisely, the labeled β_3 subunit and the labeled chimeric proteins ($[^{35}S]$ $Ca_v\beta3$, $[^{35}S]$ $Ca_v\beta3$-I-$II_{2.1}$ and $[^{35}S]$ $Ca_v\beta3$-I-$II_{2.1}\Delta AID$) are incubated in the presence or in the absence of the GST-$AID_{1.2}$ fusion protein or of the GST protein, and optionally in the presence of increasing amounts of $G\beta\gamma$ (10 to 900 nM, Calbiochem).

The incubation product is separated by polyacrylamide gel electrophoresis (SDS-PAGE) and the gel is autoradiographed.

2) Results

The results illustrated in figures 2 and 3 are as follows:

- figure 2a shows that the β_3 subunit (1 to 3 pM) binds with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein (1 µM),

- figure 2b shows that the fusion of the β_3 subunit with the I-II loop of the $\alpha_{2.1}$ subunit ($Ca_v\beta_3$-I-$II_{2.1}$ chimera) prevents its binding with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein,

- figure 2c shows that the deletion of the 18 amino acids of the $AID_{2.1}$ domain ($Ca_v\beta_3$-I-$II_{2.1}\Delta AID$ chimera) restores the binding of the β_3 subunit with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein,

- figure 3 shows that the addition of Gβγ complex displaces the intramolecular interaction between the $Ca_v\beta$ subunit and the I-II loop of the $\alpha_{2.1}$ subunit of the $Ca_v\beta_3$-I-$II_{2.1}$ chimera, thus allowing the β_3 subunit to bind with the $AID_{1.2}$ domain of the GST-$AID_{1.2}$ fusion protein; the IC_{50} concentration of Gβγ capable of displacing 50% of the binding between the $Ca_v\beta$ subunit and $AID_{2.1}$ domain, after incubation for 30 min at 30°C, is 160 nM; this value is 2 to 3 times higher than those relating to the affinity of Gβγ for the I-$II_{2.1}$ loop, previously reported (De Waard et al., Nature, 1997, 385, 446-450).

EXAMPLE 3: DEMONSTRATION *EX VIVO* OF THE DISPLACEMENT OF THE $Ca_v\alpha_{2.1}$-$Ca_v\beta$ INTERACTION BY THE G-PROTEIN Gβγ COMPLEX

1) Materials and methods

a) Cy3-labeling of the purified His-$Ca_v\beta_3$ recombinant protein

The purified His-$Ca_v\beta_3$ recombinant protein (Geib et al., Biochem J., 2002, 364, 285-292; Fathallah et al., Eur. J. Neurosci., 2002, 16, 219-228) is coupled to monoreactive Cy3 maleimide according to the supplier's instructions (Amersham Pharmacia Biotech).

b) Injection of xenopus oocytes and electrophysiological recordings

The preparation, the injection of the xenopus oocytes and the electrophysiological recordings are carried out as described in Geib et al., mentioned above. The effects of the Gβγ complexes on the current-voltage relationship and the inactivation of the equilibrium state are analyzed 30 minutes after injection.

c) Fluorescence transfer (FRET) measurement

The oocytes are analyzed by confocal microscopy (Leica TCS-SP2 microscope, in the "XYλ" mode), 4 to 7 days after injection.

The fluorescence emission is recorded using an argon laser with an excitation at 488 nm and a dichroic mirror (488/543/633). The fluorescence is measured through 14 filters (10 nm thick) so as to reconstruct the emission spectrum. For each measurement, two different regions are analyzed in order to ensure the reproducibility of the measurement. The FRET levels are estimated through the ratio (585/525) of the fluorescence at 585 nm (Cy3 acceptor emission peak) to the fluorescence at 525 nm (GFP donor emission peak).

2) <u>Results</u>

- The $Ca_v\beta_3$ subunit coupled to Cy3 (figure 4a) is as active as the $Ca_v\beta_3$ subunit on the regulation of $Ca_v\alpha_{2.1}$ channels expressed in xenopus oocytes (figure 4b).

- The injection of the Cy3-$Ca_v\beta_3$ or CFP-$Ca_v\alpha_{2.1}$ protein or else of the cDNA encoding said protein, alone or in combination, results in the emission of a high fluorescence signal at the plasma membrane (figure 5a).

Analysis of the fluorescence emission between 500 and 640 nm, after excitation at 488 nm (figure 5b), shows that GFP-$Ca_v\alpha_{2.1}$ produces a high signal with a maximum at 525 nm, whereas Cy3-$Ca_v\beta$3 alone is relatively nonexcited and produces a weak signal with a maximum at 585 nm. When the two proteins are in the oocytes, the signal emitted at 525 nm decreases drastically, whereas that at 585 nm increases significantly. These changes are readily quantifiable by determining the ratio of the fluorescence signals at 585 nm and 525 nm (R_f =

0.34 ± 0.03 for GFP-$Ca_v\alpha_{2.1}$ (n=3), R_f = 1.9 ± 0.10 for Cy3-$Ca_v\beta_3$ (n=3) and R_f = 3.9 ± 0.22 for GFP-$Ca_v\alpha_{2.1}$ + Cy3-$Ca_v\beta_3$ (n=7)). Such large changes resulting from a considerable fluorescence transfer demonstrate the proximity of the GFP-Cav$\alpha_{2.1}$ and Cy3-Cavβ3 fluorochromes.

- The injection of G$\beta\gamma$ into the ooctyes containing both GFP-Cav$\alpha_{2.1}$ and Cy3-Cavβ_3 induces a rapid disappearance of the fluorescence transfer (figure 6). By comparison, the injection of G$\beta\gamma$ has no effect in the cells containing only GFP-Cav$\alpha_{2.1}$ or Cy3-Cavβ_3.

The final ratio of the fluorescence signals (0.82 ± 0.06, n=7) is of the order of that observed in the oocytes containing only GFP-Cav$\alpha_{2.1}$ or Cy3-Cavβ_3, indicating that the dissociation of the Cy3-Cavβ_3 channel is considerable (figure 7). By comparison, the injection of heat-inactivated G$\beta\gamma$ has no effect (R_f = 3.74 ± 0.4, n=3).

These results demonstrate that G$\beta\gamma$ is as capable of displacing, *ex vivo*, the Cavβ_3 subunit from its site for binding to the Cav$\alpha_{2.1}$ channel.

EXAMPLE 4: CONSTRUCTION OF A BIOSENSOR FOR MEASURING THE ACTIVITY OF G PROTEINS BY THE FRET TECHIQUE

A chimeric protein containing a fluorescence donor fluorophore (EGFP) at its NH_2 end and a fluorescence acceptor fluorophore (CFP) at its COOH end is constructed from the vector pEYFPmemb (Clontech). This vector has the advantage of having:

- a GAP-43 sequence that makes it possible to anchor the chimera to the plasma membrane via its NH_2 end. The anchoring to the membrane has the advantage, firstly, of keeping the protein at the

membrane and, secondly, of increasing the probability of encounter between the chimeric protein and its Gβγ ligand, which is itself anchored to the membrane via binding of the palmitoylation type, and

- an EYFP sequence downstream of GAP-43.

The construction is carried out in two steps:

- 1st cloning step:

The DNA fragment encoding the GK-like domain of the β subunit (Hanlon *et al.*, FEBS, 1999, 445, 366-370) fused to the I-II loop of the α_1 subunit is amplified by PCR from the plasmid pcDNA3-$Ca_V\beta 3$-$I\text{-}II_{2.1}$ (Example 1) and is then cloned 3′ of the EYFP gene.

More precisely, the PCR amplification is carried out using the following sense and antisense primers:

BsiW I ***Pvu I***

- 5′-AGC**CGTACGCGATCG**CATCTCTAGCCAAGCAGAAGCAAA-3′

(SEQ ID NO: 6)

Hpa I ***Spe I***

5′-CCC**GTTAAC**CCC**ACTAGT**CTGAGTTTTGACCATGCGACGGAT-3′

(SEQ ID NO: 7)

The PCR product obtained is cloned between the *BsiW I* and *Hpa I* sites of the plasmid pEYFPmemb, so as to give the plasmid pEYFmemChimBeta3I-II.

- 2nd cloning step:

The cDNA encoding ECFP is amplified by PCR and then cloned into the above plasmid, 3′ of the β3-I-II insert.

More precisely, the ECFP is amplified by PCR from the vector pECFP (Clontech), using the following sense and antisense primers:

Spe I

- 5'-GGG**ACTAGT**ATGGTGAGCAAGGGCGAGGAGCTG-3' (SEQ ID NO: 8)

Hpa I

- 5'-CCC**GTTAAC**TGCCGAGAGTGATCCCGGCGGCGGT-3' (SEQ ID NO: 9)

The PCR product obtained is cloned between the *Spe I* and *Hpa I* sites of the plasmid pEYFmemChimBeta3I-II, to give the plasmid pCHIC corresponding to the sequence SEQ ID NO: 10.